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(54) Title: NOVEL HIGH AFFINITY HUMAN ANTIBODIES TO TUMOR ANTIGENS

(57) Abstract

This invention provides for novel human antibodies that specifically bind to c-erbB-2. The antibodies may be used alone or as components of chimeric molecules that specifically target and deliver effector molecules to cells overexpressing c-erbB-2.

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NOVEL HIGH AFFINITY HUMAN ANTIBODIES TO TUMOR ANTIGENS

BACKGROUND OF THE INVENTION

This application is a continuation-in-part of United States Provisional Applications U.S.S.N. 60/000,238 and U.S.S.N. 60/000,250, filed on June 14, 1995 and June 15, 1995 respectively. These applications are incorporated by reference for all purposes.

The invention was made by or under a contract with the following agencies of the United States Government: Army Grant No. DAMD17-94-J-4433 and the Department of Health and Human Services, National Institutes of Health, Grant No. U01 CA51880.

This invention pertains to the fields of immunodiagnostics and immunotherapeutics. In particular, this invention pertains to the discovery of novel human antibodies that specifically bind to c-erbB-2, and to chimeric molecules containing these antibodies.

Conventional cancer chemotherapeutic agents cannot distinguish between normal cells and tumor cells and hence damage and kill normal proliferating tissues. One approach to reduce this toxic side effect is to specifically target the chemotherapeutic agent to the tumor. This is the rationale behind the development of immunotoxins, chimeric molecules composed of an antibody either chemically conjugated or fused to a toxin that binds specifically to antigens on the surface of a tumor cell thereby killing or inhibiting the growth of the cell (Frankel *et al. Ann. Rev. Med.*, 37: 127 (1986)). The majority of immunotoxins prepared to date, have been made using murine monoclonal antibodies (Mabs) that exhibit specificity for tumor cells. Immunotoxins made from Mabs demonstrate relatively selective killing of tumor cells *in vitro* and tumor regression in animal models (*id.*).

Despite these promising results, the use of immunotoxins in humans has been limited by toxicity, immunogenicity and a failure to identify highly specific tumor antigens (Byers *et al. Cancer Res.*, 49: 6153). Nonspecific toxicity results from the

failure of the monocl nal antibody to bind specifically and with high affinity to tumor cells. As a result, nonspecific cell killing occurs. In addition, the foreign immunotoxin molecule elicits a strong immune response in humans. The immunogenicity of the toxin portion of the immunotoxin has recently been overcome by using the human analog of RNase (Rybak *et al. Proc. Nat. Acad. Sci., USA*, 89: 3165 (1992)). The murine antibody portion, however, is still significantly immunogenic (Sawler *et al.*, *J. Immunol.*, 135: 1530 (1985)).

Immunogenicity could be avoided and toxicity reduced if high affinity tumor specific human antibodies were available. However, the production of human monoclonal antibodies using conventional hybridoma technology has proven extremely difficult (James *et al.*, *J. Immunol. Meth.*, 100: 5 (1987)). Furthermore, the paucity of purified tumor-specific antigens makes it necessary to immunize with intact tumor cells or partially purified antigen. Most of the antibodies produced react with antigens which are also common to normal cells and are therefore unsuitable for use as tumor-specific targeting molecules.

SUMMARY OF THE INVENTION

This invention provides novel human antibodies that specifically bind to the extracellular domain of the c-erbB-2 protein product of the HER2/neu oncogene. This antigen (marker) is overexpressed on many cancers (*e.g.* carcinomas) and thus the antibodies of the present invention specifically bind to tumor cells that express c-erbB-2.

In a preferred embodiment, the antibody is a C6 antibody derived from the sFv antibody C6.5. The antibody may contain a variable heavy chain, a variable light chain, or both a variable heavy and variable light chain of C6.5 or its derivatives. In addition the antibody may contain a variable heavy chain, a variable light chain or both a variable heavy and variable light chain of C6.5 in which one or more of the variable heavy or variable light complementarity determining regions (CDR1, CDR2 or CDR3) has been altered (*e.g.*, mutated). Particularly preferred CDR variants are listed in the specification and in Examples 1, 2 and 3. Particularly preferred C6 antibodies include C6.5, C6ML3-14, C6L-1 and C6MH3-B1. In various preferred embodiments, these antibodies are single chain antibodies (sFv also known as scFv) comprising a variable heavy chain joined to a variable light chain either directly or through a peptide linker. Other preferred embodiments of the C6 antibodies and C6.5, C6ML3-14, C6L1, and C6MH3-B1, in particular, include Fab, the dimer $(Fab')_2$, and the dimer $(sFv')_2$.

Particularly preferred $(sFv')_2$ dimers are fusion proteins where the Sfv' components are joined through a peptide linkage or through a peptide (G_4S). Still other preferred C6 antibodies include an antibody selected from the group consisting of an antibody having a V_L domain with one of the amino acid sequences shown in Table 10, an antibody having a V_H domain with one of the amino acid sequences shown in Table 12, an antibody having a V_L CDR3 domain having one of the amino acid sequences shown in Tables 4, 15, and 16, and an antibody having a V_H CDR3 domain having one of the amino acid sequences shown in Tables 13 and 14. Other preferred embodiments are to be found replete throughout the specification.

In a particularly preferred embodiment, the C6 antibody has a K_d ranging from about 1.6×10^{-8} to about 1×10^{-12} M in SK-BR-3 cells using Scatchard analysis or as measured against purified c-erbB-2 by surface plasmon resonance in a BIAcore.

In another embodiment the present invention provides for nucleic acids that encode any of the above-described C6 antibodies. The invention also provides for nucleic acids that encode the amino acid sequences of C6.5, C6ML3-14, C6L1, C6MH3-B1, or any of the other amino acid sequences encoding C6 antibodies and described in Example 1, 2 or 3. In addition this invention provides for nucleic acid sequences encoding any of these amino acid sequences having conservative amino acid substitutions.

In still another embodiment, this invention provides for proteins comprising one or more complementarity determining regions selected from the group consisting of the complementarity determining regions of Tables 10, 12, 13, 14, 15, and 16 and of any of the examples, in particular of Examples 1, 2 or 3. Other particularly preferred antibodies include any of the antibodies expressed by the clones described herein.

In still yet another embodiment, this invention provides for cells comprising a recombinant nucleic acid which is any of the above described nucleic acids.

This invention also provides for chimeric molecules that specifically bind a tumor cell bearing c-erbB-2. The chimeric molecule comprises an effector molecule joined to any of the above-described C6 antibodies. In a preferred embodiment, the effector molecule is selected from the group consisting of a cytotoxin (e.g. PE, DT, Ricin A, *etc.*), a label, a radionuclide, a drug, a liposome, a ligand, an antibody, and an

antigen binding domaine). The C6 antibody may be chemically conjugated to the effector molecule or the chimeric molecule may be expressed as a fusion protein.

This invention provides for methods of making C6 antibodies. One method proceeds by i) providing a phage library presenting a C6.5 variable heavy chain and a multiplicity of human variable light chains; ii) panning the phage library on c-erbB-2; and iii) isolating phage that specifically bind c-erbB-2. This method optionally further includes iv) providing a phage library presenting the variable light chain of the phage isolated in step iii and a multiplicity of human variable heavy chains; v) panning the phage library on c-erbB-2; and vi) isolating phage that specifically bind c-erbB-2.

Another method for making a C6 antibody proceeds by i) providing a phage library presenting a C6.5 variable light chain and a multiplicity of human variable heavy chains; ii) panning the phage library on c-erbB-2; and iii) isolating phage that specifically bind c-erbB-2.

Yet another method for making a C6 antibody involves i) providing a phage library presenting a C6.5 variable light and a C6.5 variable heavy chain encoded by a nucleic acid variable in the sequence encoding CDR1, CDR2 or CDR3 such that each phage displays a different CDR; ii) panning the phage library on c-erbB-2; and isolating the phage that specifically bind c-erbB-2.

This invention also provides a method for impairing growth of tumor cells bearing c-erbB-2. This method involves contacting the tumor with a chimeric molecule comprising a cytotoxin attached to a human C6 antibody that specifically binds c-erbB-2.

Finally, this invention provides a method for detecting tumor cells bearing c-erbB-2. This method involves contacting the biological samples derived from a tumor with a chimeric molecule comprising a label attached to a human C6 antibody that specifically binds c-erbB-2.

Definitions

The following abbreviations are used herein: AMP, ampicillin; c-erbB-2 ECD, extracellular domain of c-erbB-2; CDR, complementarity determining region; ELISA, enzyme linked immunosorbent assay; FACS, fluorescence activated cell sorter; FR, framework region; Glu, glucose; HBS, hepes buffered saline, 10 mM hepes, 150 mM NaCl, pH 7.4; IMAC, immobilized metal affinity chromatography; k_{on}, association rate constant; k_{off}, dissociation rate constant; MPBS, skimmed milk powder in PBS;

A chimeric molecule is a molecule in which two or more molecules that exist separately in their native state are joined together to form a single molecule having the desired functionality of all of its constituent molecules. While the chimeric molecule may be prepared by covalently linking two molecules each synthesized separately, one of skill in the art will appreciate that where the chimeric molecule is a fusion protein, the chimera may be prepared de novo as a single "joined" molecule.

The term "conservative substitution" is used in reference to proteins or peptides to reflect amino acid substitutions that do not substantially alter the activity (specificity or binding affinity) of the molecule. Typically conservative amino acid substitutions involve substitution one amino acid for another amino acid with similar chemical properties (*e.g.* charge or hydrophobicity). The following six groups each contain amino acids that are typical conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the nucleic and amino acid sequence of the C6 sFv antibody C6.5.

Figure 2 shows the location of mutations in a light chain shuffled C6L1 and heavy chain shuffled C6H2 sFv. Mutations are indicated as shaded spheres on the Cα-carbon tracing of the Fv fragment of the immunoglobulin KOL (Marquart *et al.* (1990). H1, H2, H3, L1, L2 and L3 refer to the V_H and V_L antigen binding loops respectively. Mutations in C6L1 are all located in the V_L domain with parental V_H sequence, mutations in C6H2 are all located in the V_H domain with parental V_L sequence. C6L1 has no mutations located in a ß-strand which forms part of the V_H-V_L interface. C6H2 has 2 conservative mutations located in the ß-strand formed by framework 3 residues.

Figure 3 shows the locations of mutations in light chain shuffled sFv which spontaneously form dimers. Mutations are indicated as shaded spheres on the α-

carbon tracing f the Fv fragment of the immunoglobulin KOL (Marquart et al., 1980) with the V_L domain located on the left side of each panel. A=C6VLD; B=C6VLE; C=CdVLB; D=C6VLF. Each shuffled sFv has at least 1 mutation located in a ß-strand which forms part of the V_H-V_L interface.

Figure 4 illustrates the 72 hour biodistribution of a C6.5 diabody in SK-OV-3 tumor-bearing scid mice.

DETAILED DESCRIPTION

This invention provides for novel human antibodies that specifically bind to the extracellular domain of the c-erbB-2 protein product of the HER2/neu oncogene. The c-erbB-2 marker is overexpressed by 30-50% of breast carcinomas and other adenocarcinomas and thus provides a suitable cell surface marker for specifically targeting tumor cells such as carcinomas. In contrast to previous known anti-cerbB-2 antibodies, the antibodies of the present invention (designated herein as C6 antibodies) are fully human antibodies. Thus, administration of these antibodies to a human host elicits a little or no immunogenic response.

This invention additionally provides for chimeric molecules comprising the C6 antibodies of the present invention joined to an effector molecule. The C6 antibodies act as a "targeting molecule" that serves to specifically bind the chimeric molecule to cells bearing the c-erbB-2 marker thereby delivering the effector molecule to the target cell.

An effector molecule typically has a characteristic activity that is desired to be delivered to the target cell (*e.g.* a tumor overexpressing c-erbB-2). Effector molecules include cytotoxins, labels, radionuclides, ligands, antibodies, drugs, liposomes, and viral coat proteins that render the virus capable of infecting a c-erbB-2 expressing cell. Once delivered to the target, the effector molecule exerts its characteristic activity.

For example, in one embodiment, where the effector molecule is a cytotoxin, the chimeric molecule acts as a potent cell-killing agent specifically targeting the cytotoxin to tumor cells bearing the c-erbB-2 marker. Chimeric cytotoxins that specifically target tumor cells are well known to those of skill in the art (*see, for example*, Pastan *et al., Ann. Rev. Biochem.*, 61: 331-354 (1992)).

In another embodiment, the chimeric molecule may be used for detecting the presence or absence of tumor cells *in vivo* or *in vitro* or for localizing tumor cells *in*

vivo. These methods involve providing a chimeric molecule comprising an effector molecule, that is a detectable label attached to the C6 antibody. The C6 antibody specifically binds the chimeric molecule to tumor cells expressing the c-erbB-2 marker which are then marked by their association with the detectable label. Subsequent detection of the cell-associated label indicates the presence and/or location of a tumor cell.

In yet another embodiment, the effector molecule may be another specific binding moiety including, but not limited to an antibody, an antigen binding domain, a growth factor, or a ligand. The chimeric molecule will then act as a highly specific bifunctional linker. This linker may act to bind and enhance the interaction between cells or cellular components to which the chimeric protein binds. Thus, for example, where the "effector" component is an anti-receptor antibody or antibody fragment, the C6 antibody component specifically binds c-erbB-2 bearing cancer cells, while the effector component binds receptors (*e.g.*, IL-2, IL-4, FcγI, FcγII and FcγIII receptors) on the surface of immune cells. The chimeric molecule may thus act to enhance and direct an immune response toward target cancer cells.

In still yet another embodiment the effector molecule may be a pharmacological agent (*e.g.* a drug) or a vehicle containing a pharmacological agent. This is particularly suitable where it is merely desired to invoke a non-lethal biological response. Thus the C6 antibody receptor may be conjugated to a drug such as vinblastine, vindesine, melphalan, N-Acetylmelphalan, methotrexate, aminopterin, doxirubicin, daunorubicin, genistein (a tyrosine kinase inhibitor), an antisense molecule, and other pharmacological agents known to those of skill in the art, thereby specifically targeting the pharmacological agent to tumor cells expressing c-erbB-2.

Alternatively, the C6 antibody may be bound to a vehicle containing the therapeutic composition. Such vehicles include, but are not limited to liposomes, micelles, various synthetic beads, and the like.

One of skill in the art will appreciate that the chimeric molecules of the present invention optionally includes multiple targeting moieties bound to a single effector or conversely, multiple effector molecules bound to a single targeting moiety. In still other embodiment, the chimeric molecules includes both multiple targeting moieties and multiple effector molecules. Thus, for example, this invention provides for "dual targeted" cytotoxic chimeric molecules in which the C6 antibody is attached to a

cytotoxic molecule while another molecule (*e.g.* an antibody, or another ligand) is attached to the other terminus of the toxin. Such a dual-targeted cytotoxin might comprise, *e.g.* a C6 antibody substituted for domain Ia at the amino terminus of a PE and anti-TAC(Fv) inserted in domain III. Other antibodies may also be suitable effector molecules.

I. Preparation of C6 Antibodies.

The C6 antibodies of this invention are prepared using standard techniques well known to those of skill in the art in combination with the polypeptide and nucleic acid sequences provided herein. The polypeptide sequences may be used to determine appropriate nucleic acid sequences encoding the particular C6 antibody disclosed thereby. The nucleic acid sequence may be optimized to reflect particular codon "preferences" for various expression systems according to standard methods well known to those of skill in the art. Alternatively, the nucleic acid sequences provided herein may also be used to express C6 antibodies.

Using the sequence information provided, the nucleic acids may be synthesized according to a number of standard methods known to those of skill in the art. Oligonucleotide synthesis, is preferably carried out on commercially available solid phase oligonucleotide synthesis machines (Needham-VanDevanter *et al.* (1984) *Nucleic Acids Res.* 12:6159-6168) or manually synthesized using the solid phase phosphoramidite triester method described by Beaucage *et. al.* (Beaucage *et. al.* (1981) *Tetrahedron Letts.* 22(20): 1859-1862).

Once a nucleic acid encoding a C6 antibody is synthesized it may be amplified and/or cloned according to standard methods. Molecular cloning techniques to achieve these ends are known in the art. A wide variety of cloning and *in vitro* amplification methods suitable for the construction of recombinant nucleic acids, *e.g.*, encoding C6 antibody genes, are known to persons of skill. Examples of these techniques and instructions sufficient to direct persons of skill through many cloning exercises are found in Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology* volume 152 Academic Press, Inc., San Diego, CA (Berger); Sambrook *et al.* (1989) *Molecular Cloning - A Laboratory Manual* (2nd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY, (Sambrook); and *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, eds., Current Protocols, a

joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1994 Supplement) (Ausubel). Methods of producing recombinant immunoglobulins are also known in the art. *See*, Cabilly, U.S. Patent No. 4,816,567; and Queen *et al.* (1989) *Proc. Nat'l Acad. Sci. USA* 86: 10029-10033.

Examples of techniques sufficient to direct persons of skill through *in vitro* amplification methods, including the polymerase chain reaction (PCR) the ligase chain reaction (LCR), Qβ-replicase amplification and other RNA polymerase mediated techniques are found in Berger, Sambrook, and Ausubel, as well as Mullis *et al.*, (1987) U.S. Patent No. 4,683,202; *PCR Protocols A Guide to Methods and Applications* (Innis *et al.* eds) Academic Press Inc. San Diego, CA (1990) (Innis); Arnheim & Levinson (October 1, 1990) *C&EN* 36-47; *The Journal Of NIH Research* (1991) 3, 81-94; (Kwoh *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86, 1173; Guatelli *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87, 1874; Lomell *et al.* (1989) *J. Clin. Chem* 35, 1826; Landegren *et al.*, (1988) *Science* 241, 1077-1080; Van Brunt (1990) *Biotechnology* 8, 291-294; Wu and Wallace, (1989) *Gene* 4, 560; and Barringer *et al.* (1990) *Gene* 89, 117. Improved methods of cloning *in vitro* amplified nucleic acids are described in Wallace *et al.*, U.S. Pat. No. 5,426,039.

Once the nucleic acid for a C6 antibody is isolated and cloned, one may express the gene in a variety of recombinantly engineered cells known to those of skill in the art. Examples of such cells include bacteria, yeast, filamentous fungi, insect (especially employing baculoviral vectors), and mammalian cells. It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for expression of C6 antibodies.

In brief summary, the expression of natural or synthetic nucleic acids encoding C6 antibodies will typically be achieved by operably linking a nucleic acid encoding the antibody to a promoter (which is either constitutive or inducible), and incorporating the construct into an expression vector. The vectors can be suitable for replication and integration in prokaryotes, eukaryotes, or both. Typical cloning vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the nucleic acid encoding the C6 antibody. The vectors optionally comprise generic expression cassettes containing at least one independent terminator sequence, sequences permitting replication of the cassette in both

eukaryotes and prokaryotes, *i.e.*, shuttle vectors, and selection markers for both prokaryotic and eukaryotic systems. *See* Sambrook.

To obtain high levels of expression of a cloned nucleic acid it is common to construct expression plasmids which typically contain a strong promoter to direct transcription, a ribosome binding site for translational initiation, and a transcription/translation terminator. Examples of regulatory regions suitable for this purpose in *E. coli* are the promoter and operator region of the *E. coli* tryptophan biosynthetic pathway as described by Yanofsky, C., 1984, *J. Bacteriol.*, 158:1018-1024 and the leftward promoter of phage lambda (P_L) as described by Herskowitz and Hagen, 1980, *Ann. Rev. Genet.*, 14:399-445. The inclusion of selection markers in DNA vectors transformed in *E. coli* is also useful. Examples of such markers include genes specifying resistance to ampicillin, tetracycline, or chloramphenicol. *See* Sambrook for details concerning selection markers, *e.g.*, for use in *E. coli*.

Expression systems for expressing C6 antibodies are available using *E. coli*, *Bacillus sp.* (Palva *et al.* (1983) *Gene* 22:229-235; Mosbach *et al.*, *Nature*, 302:543-545 and *Salmonella*. *E. coli* systems are preferred.

The C6 antibodies produced by prokaryotic cells may require exposure to chaotropic agents for proper folding. During purification from, *e.g.*, *E. coli*, the expressed protein is optionally denatured and then renatured. This is accomplished, *e.g.*, by solubilizing the bacterially produced antibodies in a chaotropic agent such as guanidine HCl. The antibody is then renatured, either by slow dialysis or by gel filtration. *See*, U.S. Patent No. 4,511,503.

Methods of transfecting and expressing genes in mammalian cells are known in the art. Transducing cells with nucleic acids can involve, for example, incubating viral vectors containing C6 nucleic acids with cells within the host range of the vector. See, *e.g.*, *Methods in Enzymology*, vol. 185, Academic Press, Inc., San Diego, CA (D.V. Goeddel, ed.) (1990) or M. Krieger, *Gene Transfer and Expression — A Laboratory Manual*, Stockton Press, New York, NY, (1990) and the references cited therein.

The culture of cells used in the present invention, including cell lines and cultured cells from tissue or blood samples is well known in the art. Freshney (*Culture of Animal Cells, a Manual of Basic Technique, third edition* Wiley-Liss, New York (1994)) and the references cited therein provides a general guide to the culture of cells.

Techniques for using and manipulating antibodies are found in Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; Harlow and Lane (1989) *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY; Stites *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY; and Kohler and Milstein (1975) Nature 256: 495-497. C6 antibodies which are specific for *c-erbB-2* bind *c-erbB-2* and have a K_D of 1μM or better, with preferred embodiments having a K_D of 1 nM or better and most preferred embodiments having a K_D of 0.1 nM or better.

In a preferred embodiment the C6 antibody gene (*e.g.* C6.5 sFv gene) is subcloned into the expression vector pUC119Sfi/NotHismyc, which is identical to the vector described by Griffiths *et al.*, *EMBO J.*, 13: 3245-3260 (1994), (except for the elimination of an XBaI restriction site). This results in the addition of a hexa-histidine tag at the C-terminal end of the sFv. A pHEN-1 vector DNA containing the C6.5 sFv DNA is prepared by alkaline lysis miniprep, digested with NcoI and NotI, and the sFv DNA purified on a 1.5% agarose gel. The C6 sFv DNA is ligated into pUC119Sfi1/Not1Hismyc digested with NcoI and NotI and the ligation mixture used to transform electrocompetent *E.coli* HB2151. For expression, 200 ml of 2 x TY media containing 100 mg/ml ampicillin and 0.1% glucose is inoculated with *E.coli* HB2151 harboring the C6 gene in pUC119Sfi1/Not1Hismyc. The culture is grown at 37°C to an A600 nm of 0.8. Soluble sFv is expression induced by the addition of IPTG to a final concentration of 1 mM, and the culture is grown at 30° C in a shaker flask overnight.

The C6 sFv may then be harvested from the periplasm using the following protocol: Cells are harvested by centrifugation at 4000 g for 15 min, resuspended in 10 ml of ice cold 30 mM Tris-HCl pH 8.0, 1 mM EDTA, 20% sucrose, and incubated on ice for 20 minutes. The bacteria are then pelleted by centrifugation at 6000 g for 15 min. and the "periplasmic fraction" cleared by centrifugation at 30,000g for 20 min. The supernatant is then dialyzed overnight at 4°C against 8 L of IMAC loading buffer (50 mM sodium phosphate pH 7.5, 500 mM NaCl, 20 mM imidazole) and then filtered through a 0.2 micron filter.

In a preferred embodiment, the C6 sFv is purified by IMAC. All steps are performed at 4°C. A column containing 2 ml of Ni-NTA resin (Qiagen) is washed with 20 ml IMAC column wash buffer (50 mM sodium ph sphate pH 7.5, 500 mM

NaCl, 250 mM imidazole) and 20 ml of IMAC loading buffer. The periplasmic preparation is then loaded onto the column and the column washed sequentially with 50 ml IMAC loading buffer and 50 ml IMAC washing buffer (50 mM sodium phosphate pH 7.5, 500 mM NaCl, 25 mM imidazole). Protein was eluted with 25 ml IMAC elution buffer (50 mM sodium phosphate pH 7.5, 500 mM NaCl, 100 mM imidazole) and 4 ml fractions collected. The C6 antibody may be detected by absorbance at 280 nm and sFv fraction eluted. To remove dimeric and aggregated sFv, samples can be concentrated to a volume < 1 ml in a Centricon 10 (Amicon) and fractionated on a Superdex 75 column using a running buffer of HBS (10 mM Hepes, 150 mM NaCl, pH 7.4).

The purity of the final preparation may be evaluated by assaying an aliquot by SDS-PAGE. The protein bands can be detected by Coomassie staining. The concentration can then be determined spectrophotometrically, assuming that an A_{280} nm of 1.0 corresponds to an sFv concentration of 0.7 mg/ml .

II. Modification of C6 Antibodies.

A) Display of antibody fragments on the surface of bacteriophage (phage display).

Display of antibody fragments on the surface of viruses which infect bacteria (bacteriophage or phage) makes it possible to produce human sFvs with a wide range of affinities and kinetic characteristics. To display antibody fragments on the surface of phage (phage display), an antibody fragment gene is inserted into the gene encoding a phage surface protein (pIII) and the antibody fragment-pIII fusion protein is expressed on the phage surface (McCafferty *et al.* (1990) *Nature*, 348: 552-554; Hoogenboom *et al.* (1991) *Nucleic Acids Res.*, 19: 4133-4137). For example, a sFv gene coding for the V_H and V_L domains of an anti-lysozyme antibody (D1.3) was inserted into the phage gene III resulting in the production of phage with the D1.3 sFv joined to the N-terminus of pIII thereby producing a "fusion" phage capable of binding lysozyme (McCafferty *et al.* (1990) *Nature*, 348: 552-554).

Since the antibody fragments on the surface of the phage are functional, phage bearing antigen binding antibody fragments can be separated from non-binding or lower affinity phage by antigen affinity chromatography (McCafferty *et al.* (1990) *Nature*, 348: 552-554). Mixtures of phage are allowed to bind to the affinity matrix, non-binding or lower affinity phage are removed by washing, and bound phage are eluted

by treatment with acid r alkali. Depending on the affinity of the antibody fragment, enrichment factors of 20 fold-1,000,000 fold are obtained by single round of affinity selection. By infecting bacteria with the eluted phage, however, more phage can be grown and subjected to another round of selection. In this way, an enrichment of 1000 fold in one round becomes 1,000,000 fold in two rounds of selection (McCafferty *et al.* (1990) *Nature*, 348: 552-554). Thus, even when enrichments in each round are low (Marks *et al.* (1991) *J. Mol. Biol.*, 222: 581-597), multiple rounds of affinity selection leads to the isolation of rare phage and the genetic material contained within which encodes the sequence of the binding antibody. The physical link between genotype and phenotype provided by phage display makes it possible to test every member of an antibody fragment library for binding to antigen, even with libraries as large as 100,000,000 clones. For example, after multiple rounds of selection on antigen, a binding sFv that occurred with a frequency of only 1/30,000,000 clones was recovered (Marks *et al.* (1991) *J. Mol. Biol.*, 222: 581-597).

Analysis of binding is simplified by including an amber codon between the antibody fragment gene and gene III. This makes it possible to easily switch between displayed and soluble antibody fragments simply by changing the host bacterial strain. When phage are grown in a supE suppresser strain of *E. coli*, the amber stop codon between the antibody gene and gene III is read as glutamine and the antibody fragment is displayed on the surface of the phage. When eluted phage are used to infect a non-supressor strain, the amber codon is read as a stop codon and soluble antibody is secreted from the bacteria into the periplasm and culture media (Hoogenboom *et al.* (1991) *Nucleic Acids Res.*, 19: 4133-4137). Binding of soluble sFv to antigen can be detected, *e.g.*, by ELISA using a murine IgG monoclonal antibody (*e.g.*, 9EIO) which recognizes a C-terminal *myc* peptide tag on the sFv (Evan *et al.* (1985) *Mol. Cell Biol.*, 5: 3610-3616; Munro *et al.* (1986) *Cell*, 46: 291-300), *e.g.*, followed by incubation with polyclonal anti-mouse Fc conjugated to horseradish peroxidase.

<u>B) Phage display can be used to increase antibody affinity.</u>

To create higher affinity antibodies, mutant sFv gene repertories, based on the sequence of a binding sFv, are created and expressed on the surface of phage. Higher affinity sFvs are selected on antigen as described above and in Examples 1 and 2. One approach for creating mutant sFv gene repertoires has been to replace either the V_H

or V_L gene from a binding sFv with a repertoire of nonimmune V_H or V_L genes (chain shuffling) (Clackson *et al.* (1991) *Nature*, 352: 624-628). Such gene repertoires contain numerous variable genes derived from the same germline gene as the binding sFv, but with point mutations (Marks *et al.* (1992) *Bio/Technology*, 10: 779-783). Using light chain shuffling and phage display, the binding avidities of a human sFv antibody fragment can be dramatiaclly increased. *See, e.g.*, Marks *et al. Bio/Technology*, 10: 779-785 (1992) in which the affinity of a human sFv antibody fragment which bound the hapten phenyloxazolone (phox) was increased from 300 nM to 15 nM (20 fold) (Marks *et al.* (1992) *Bio/Technology*, 10: 779-783).

C) Isolation and characterization of C6.5, a human sFv which binds c-erbB-2.

Isolation and characterization of C6.5 is described in detail in the Examples below. Human sFvs which bound to c-erbB-2 were isolated by selecting the nonimmune human sFv phage antibody library (described in Example 1) on c-erbB-2 extracellular domain peptide immobilized on polystyrene. After five rounds of selection, 45 of 96 clones analyzed (45/96) produced sFv which bound c-erb-B2 by ELISA. Restriction fragments analysis and DNA sequencing revealed the presence of two unique human sFvs, C4 and C6.5. Both of these sFvs bound only to c-erbB-2 and not to a panel of 10 irrelevant antigens. Cell binding assays, however, indicated that only C6.5 bound c-erb-B2 expressed on cells, and thus this sFv was selected for further characterization.

D) Purification of C6.5.

To facilitate purification, the C6.5 sFv gene was subcloned into the expression vector pUC119 Sfi-NotmycHIS which results in the addition of the myc peptide tag followed by a hexahistidine tag at the C-terminal end of the sFv. The vector also encodes the pectate lyase leader sequence which directs expression of the sFv into the bacterial periplasm where the leader sequence is cleaved. This makes it possible to harvest native properly folded sFv directly from the bacterial periplasm. Native C6.5 sFv was expressed and purified from the bacterial supernatant using immobilized metal affinity chromatography. The yield after purification and gel filtration on a Superdex 75 column was 10.5 mg/L. Other C6 antibodies may be purified in a similar manner.

V. Pharmaceutical Compositions.

The chimeric molecules of this invention are useful for parenteral, topical, oral, or local administration, such as by aerosol or transdermally, for prophylactic and/or therapeutic treatment. The pharmaceutical compositions can be administered in a variety of unit dosage forms depending upon the method of administration. For example, unit dosage forms suitable for oral administration include powder, tablets, pills, capsules and lozenges. It is recognized that the fusion proteins and pharmaceutical compositions of this invention, when administered orally, must be protected from digestion. This is typically accomplished either by complexing the protein with a composition to render it resistant to acidic and enzymatic hydrolysis or by packaging the protein in an appropriately resistant carrier such as a liposome. Means of protecting proteins from digestion are well known in the art.

The pharmaceutical compositions of this invention are particularly useful for parenteral administration, such as intravenous administration or administration into a body cavity or lumen of an organ. The compositions for administration will commonly comprise a solution of the chimeric molecule dissolved in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, *e.g.*, buffered saline and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well known sterilization techniques.. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of chimeric molecule in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs.

Thus, a typical pharmaceutical composition for intravenous administration would be about 0.1 to 10 mg per patient per day. Dosages from 0.1 up to about 100 mg per patient per day may be used, particularly when the drug is administered to a secluded site and not into the blood stream, such as into a body cavity or into a lumen of an organ. Methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in such publications

as *Remington's Pharmaceutical Science*, 15th ed., Mack Publishing Company, Easton, Pennsylvania (1980).

The compositions containing the present fusi n proteins or a cocktail thereof (*i.e.*, with other proteins) can be administered for therapeutic treatments. In therapeutic applications, compositions are administered to a patient suffering from a disease, typically a c-erbB-2 positive carcinoma, in an amount sufficient to cure or at least partially arrest the disease and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the disease and the general state of the patient's health.

Single or multiple administrations of the compositions may be administered depending on the dosage and frequency as required and tolerated by the patient. In any event, the composition should provide a sufficient quantity of the proteins of this invention to effectively treat the patient.

Among various uses of the cytotoxic fusion proteins of the present invention are included a variety of disease conditions caused by specific human cells that may be eliminated by the toxic action of the protein. One application is the treatment of cancer, such as by the use of a C6 antibody attached to a cytotoxin.

Another approach involves using a ligand that binds a cell surface marker (receptor) so the chimeric associates cells bearing the ligand substrate are associated with the c-erbB-2 overexpressing tumor cell. The ligand portion of the molecule is chosen according to the intended use. Proteins on the membranes of T cells that may serve as targets for the ligand includes FcγI, FcγII and FcγIII, CD2 (T11), CD3, CD4 and CD8. Proteins found predominantly on B cells that might serve as targets include CD10 (CALLA antigen), CD19 and CD20. CD45 is a possible target that occurs broadly on lymphoid cells. These and other possible target lymphocyte target molecules for the chimeric molecules bearing a ligand effector are described in *Leukocyte Typing III*, A.J. McMichael, ed., Oxford University Press (1987). Those skilled in the art will realize ligand effectors may be chosen that bind to receptors expressed on still other types of cells as described above, for example, membrane glycoproteins or ligand or hormone receptors such as epidermal growth factor receptor and the like.

WHAT IS CLAIMED IS:

1. A human antibody that specifically binds to c-erbB-2, said antibody being a C6 antibody.

2. The antibody of claim 1, wherein said antibody has the variable heavy (V_H) chain of C6.5.

3. The antibody of claim 1, wherein said antibody has the variable light (V_H) chain of C6.5.

4. The antibody of claim 1, wherein said antibody is C6.5.

5. The antibody of claim 1, wherein said antibody has the amino acid sequence of C6.5.

6. The antibody of claim 1, wherein said antibody has the amino acid sequence of C6ML3-14.

7. The antibody of claim 1, wherein said antibody has the amino acid sequence of C6L1.

8. The antibody of claim 1, wherein said antibody has the amino acid sequence of C6MH3-B1.

9. The antibody of claim 1, wherein said antibody has the amino acid sequence of C6ML3-9.

10. The antibody of claim 1, wherein said antibody is selected from the group consisting of an antibody having a V_L domain with one of the amino acid sequences shown in Table 10, an antibody having a V_H domain with one of the amino acid sequences shown in Table 12, an antibody having a V_L CDR3 domain having one of

the amin acid sequences shown in Tables 4, 15, and 16, and an antibody having a V_H CDR3 domain having one f the amino acid sequences shown in Tables 13 and 14.

11. The antibody of claim 1, wherein said antibody expressed by any of the clones listed in Table 16.

12. The antibody of claim 1, wherein said antibody is an Fab.

13. The antibody of claim 1, wherein said antibody is an $(Fab')_2$.

14. The antibody of claim 1, wherein said antibody$(sFv')_2$.

15. The antibody of claim 14, wherein said $(Sfv')_2$ is a fusion protein of two sFv' fragments.

16. The antibody of claim 1, wherein said antibody is C6.5 Fab.

17. The antibody of claim 1, wherein said antibody is C6.5$(Fab')_2$.

18. The antibody of claim 1, wherein said antibody is C6.5$(sFv')_2$.

19. The antibody of claim 1, wherein said antibody has a K_d ranging from about 1.6×10^{-8} M to 1.0×10^{-11} M in SK-BR-3 using a Scatchard assay or against purified c-erbB-2 by surface plasmon resonance in a BIAcore.

20. The antibody of claim 19, wherein said K_d is about 1.6×10^{-8} M.

21. A nucleic acid encoding a human C6 antibody that specifically binds to c-erbB-2.

22. The nucleic acid of claim 21, wherein said C6 antibody binds to SK-BR-3 cells with a K_d less than about 1.6×10^{-8} as determined using a scatchard assay.

23. The nucleic acid of claim 21, wherein said nucleic acid encodes an an antibody selected from the group consisting of an antibody having a 1V_L domain containing one of the amino acid sequences shown in Table 10, an antibody having a V_H domain containing one of the amino acid sequences shown in Table 12, an antibody having a V_L CDR3 domain containing one of the amino acid sequences shown in Tables 4, 15, and 16, and an antibody having a V_H CDR3 domain containing one of the amino acid sequences shown in Tables 13 and 14.

24. The nucleic acid of claim 21, wherein said nucleic acid encodes the variable light (V_L) chain of C6.5.

25. The nucleic acid of claim 21, wherein said nucleic acid encodes the variable heavy (V_H) chain of C6.5.

26. The nucleic acid of claim 21, wherein said nucleic acid encodes C6.5.

27. The nucleic acid of claim 21, wherein said nucleic acid encodes the the amino acid of a C6.5 antibody and conservative amino acid substitutions of said C6.5 antibody.

28. A cell comprising a recombinant nucleic acid that encodes a human antibody that specifically binds c-erbB-2, wherein said antibody is a C6 antibody.

29. A chimeric molecule that specifically binds a tumor cell bearing c-erbB-2, said chimeric molecule comprising an effector molecule attached to a human C6 antibody that specifically binds c-erbB-2.

30. The chimeric molecule of claim 28, wherein said C6 antibody is a single chain Fv (sFv).

31. The chimeric molecule of claim 28, wherein said effector molecule is selected from the group consisting of a cytotoxin, a label, a radionuclide, a drug, a liposome, a ligand, and an antibody.

32. The chimeric molecule of claim 28, wherein said effector molecule is a *Pseudomonas* exotoxin.

33. The chimeric molecule of claim 28, wherein said chimeric molecule is a fusion protein.

34. A method for making a C6 antibody, said method comprising:

i) providing a phage library presenting a C6.5 variable heavy (V_H) chain and a multiplicity of human variable light (V_L) chains;

ii) panning said phage library on c-erbB-2; and

iii) isolating phage that specifically bind said c-erbB-2.

35. The method of claim 33, further comprising:

iv) providing a phage library presenting a the variable light chain (V_L) of the phage isolated in step iii and a multiplicity of human variable heavy (V_H) chains;

v) panning said phage library on immobilized c-erbB-2; and

vi) isolating phage that specifically bind said c-erbB-2.

36. A method for making a C6 antibody, said method comprising:

i) providing a phage library presenting a C6.5 variable light (V_L) chain and a multiplicity of human variable heavy (V_H) chains;

ii) panning said phage library on immobilized c-erbB-2; and

iii) isolating phage that specifically bind said c-erbB-2.

37. A method for making a C6 antibody, said method comprising:

i) providing a phage library presenting a C6.5 variable light (V_L) and a C6.5 variable heavy chain encoded by a nucleic acid variable in the sequence encoding the CDRs such that each phage display a different CDR;

ii) panning said phage library on c-erbB-2; and

iii) isolating phage that specifically bind said c-erbB-2.

38. A method for impairing growth of tumor cells bearing c-erbB-2, said method comprising contacting said tumor with a chimeric molecule comprising a cytotoxin attached to a human C6 antibody that specifically binds c-erbB-2.

39. A method for detecting tumor cells bearing c-erbB-2, said method comprising contacting said tumor with a chimeric molecule comprising a label attached to a human C6 antibody that specifically binds c-erbB-2.

40. A polypeptide comprising one or more of the complementarity determining regions (CDRs) whose amino acid sequence contains a CDR sequence selected from the group consisting of the CDRs listed in Tables 4, 10, 12, 13, 14, 15, and 16.

41. A nucleic acid molecule comprising a nucleotide sequence encoding a single chain polypeptide that exhibits the antibody-binding specificity of a human C6 antibody, said polypeptide comprising:

a) a first polypeptide domain, comprising an amino acid sequence that is the binding portion of a variable region of a heavy chain of a human C6 antibody;

b) a second polypeptide domain, comprising an amino acid sequence that is the binding portion of a variable region of a light chain of a human C6 antibody; and

c) at least one polypeptide linkers comprising an amino acid sequence spanning the distance between the C-terminus of one of the first or second domains and the N-terminus of the other, whereby said linker joins the first and second polypeptide domains into a single chain polypeptide.

42. A polypeptide which exhibits immunological binding properties of a human C6 antibody, said polypeptide comprising first and second domains connected by a linker moiety, wherein:

a) the first domain comprises at least one amino acid sequence that is a CDR derived from a heavy chain of a human C6 antibody; and

b) the second domain comprises at least one amino acid sequence that is a CDR derived from a light chain of a human C6 antibody.

43. The polypeptide of claim 42, wherein the first domain comprises a a heavy chain of a human C6 antibody.

44. The polypeptide of claim 42, wherein the second domain comprises a light chain of a human C6 antibody.

45. An expression cassette, comprising:

a) the nucleic acid molecule of claim 41; and

b) a control sequence operably linked to the nucleic molecule and capable of directing the expression thereof.

46. An expression cassette, comprising:

a) the nucleic acid molecule of claim 41; and

b) a control sequence operably linked to the nucleic molecule and capable of directing the expression thereof.

47. An expression cassette, comprising:

a) the nucleic acid molecule of claim 41; and

b) a control sequence operably linked to the nucleic molecule and capable of directing the expression thereof.

48. A method of inducing the production of a polypeptide, comprising:

a) introducing the expression cassette of claim 47 into a host cell whereby the cassette is compatible with the host cell and replicates in the host cell;

b) growing the host cell whereby the polypeptide is expressed; and

c) isolating the polypeptide.

49. A method of inducing the production of a polypeptide, comprising:

a) introducing the expression cassette of claim 47 into a host cell whereby the cassette is compatible with the host cell and replicates in the host cell;

b) growing the host cell whereby the polypeptide is expressed; and

c) isolating the polypeptide.

50. A method of inducing the production of a polypeptide, comprising:

a) introducing the expression cassette of claim 47 into a host cell whereby the cassette is compatible with the host cell and replicates in the host cell;

b) growing the host cell whereby the polypeptide is expressed; and

c) isolating the polypeptide.